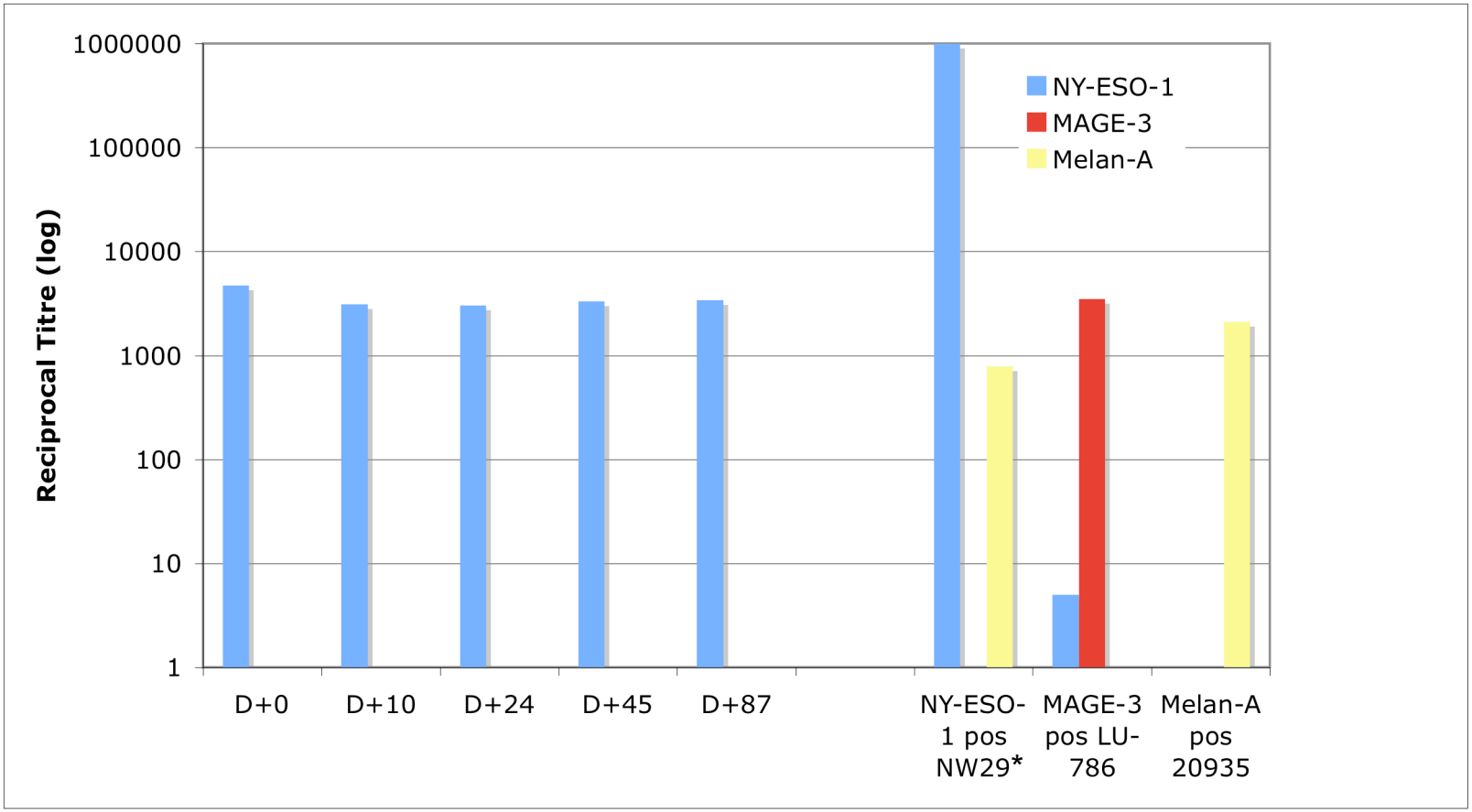
Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Hunder NN, Wallen H, Cao J, et al. Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. N Engl J Med 2008;358:2698-703.

Supplemental Figure 1



(* NW29 expresses NY-ESO-1 and Melan-A)

Supplemental Figure 1

Serologic response to NY-ESO-1, MAGE-3, and MART-1/Melan-A.

Peripheral plasma samples collected pre-infusion (D+0) and post-infusion through to Day 87 were analyzed for IgG responses to recombinant NY-ESO-1, MAGE-A3, and MART-1/Melan-A proteins. Controls, using NY-ESO-1 and/or MART-1/Melan-A reactive sera from melanoma patients NW29 and 20935 as well as MAGE-A3 reactive plasma from non-small cell lung cancer patient LU-786, confirmed the specificity and reactivity of ELISA. Significant antibody response to NY-ESO-1 is observed pre-infusion but no response to MAGE-3 or MART-1/Melan-A; these titers do not change appreciably following adoptive transfer of NY-ESO-1 specific CD4 T cells.

Supplemental Data on NY-ESO-1-specific CD4 T cell clone (clone#56) .

Generation of NY-ESO-1 specific CD4 T cell clone:

Peripheral blood mononuclear cells (PBL) were obtained from the patient with melanoma by leukapheresis. PBL were thawed and resuspended in AIM-V medium at $3x10^6$ cells/mL, and then placed in sterile tissue culture dishes at 3mL/well for separation into adherent and non-adherent populations by culture for 1 hour at 37°C. The adherent cells were treated with GM-CSF (Endogen) and IL-4 (Endogen) for 6 days at which time, the PBL-derived dendritic cells (DC) growing in the culture were matured for 2 days using cytokine cocktail. Excess DC were frozen to be used for 2nd and 3rd stimulations.

$1.8x10^6$ DC were pulsed with 40 mcg/mL of GMP-certified peptide at a cell concentration of $2x10^6$/mL in PBS with 1% HSA. The DC are gamma-irradiated, washed, and resuspended in medium containing RPMI, 25mM HEPES, 2mM L-glutamine, and 10% human AB serum (CTL medium). DC were then added to $70x10^6$ PBL and plated in 48-well tissue culture treated plates at 1mL/well. Cultures were incubated for seven days at 37°C in 5.0% CO2. The cultures were restimulated once to twice with gamma-irradiated stimulator cells (thawed DC or monocytes) and pulsed with 10 mcg/mL of peptide.

All cell lines were tested by proliferation assay and ELISA for specific activity against peptide-pulsed and unpulsed targets, antigen-negative and antigen-positive tumor prior to T cell cloning to confirm the presence of antigen-specific activity. T cells showing from lines showing specific reactivity were plated at limiting dilution in 96-well round-bottomed plates. Each well received 30 ng/mL of anti-CD3 (OKT3, ORTHOCLONE), 7.5×10^4 gamma irradiated (4500 rads) allogeneic PBL and 1×10^4 gamma irradiated (8000 rads) allogeneic LCL as feeder cells in 0.2 mL of culture media containing 50 units IL-2 per mL. Cryopreserved allogeneic cells for feeder cells were obtained by leukapheresis of donors screened by the standard tests for donated blood at the Puget Sound Blood Center. Allogeneic LCL (or EBV-transformed lymphoblastoid cell lines) were derived from the TM-LCL line which has been approved by the FDA for use in adoptive therapy.

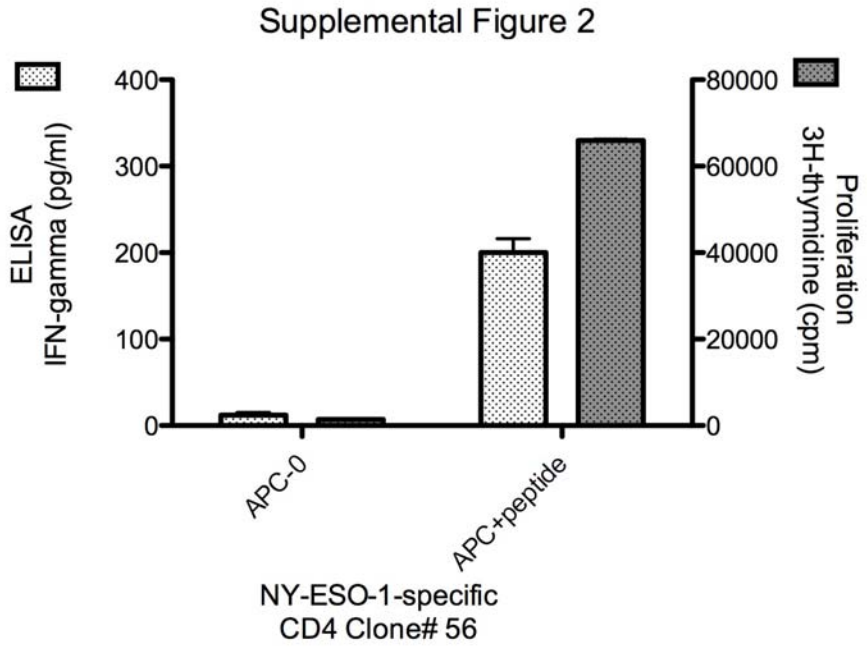
Wells positive for growth were identified 14 -21 days after plating. Those clones demonstrating MHC-restricted activity for antigen-expressing target cells were identified, restimulated and expanded in CTL medium in 25cm2 flasks with anti-CD3 monoclonal antibody and irradiated allogeneic PBL and LCL added as feeder cells. The cultures were fed with IL-2 50 U/mL 24 hours after restimulation, and every 48 to 96 hours thereafter.

Specificity Data on CD4 T cell clone# 56 used for patient infusion

Among 192 clones tested by IFN-gamma ELISA, 4 clones were selected on the basis of superior replicative potential (fold-expansion) and specific reactivity to NY-ESO-1. These clones were expanded from cloning plates and tested again by ELISA and thymidine incorporation.

When evaluated by IFN-gamma production, autologous antigen-presenting cells (LCLs) are pulsed with 10 ucg/ml of the DP4-restricted epitope peptide of NY-ESO-1 and co-cultivated with T cells at a stimulator: responder ratio of 2:1. Supernatant harvested after 18 hours is collected and analyzed by ELISA for IFN-gamma release. The IFN-gamma ELISA shows that the clone produces 200pg/mL INF-gamma when stimulated with APCs pulsed with NY-ESO-1 peptide (APC+peptide), approximately 17x more INF-gamma than T-cells stimulated with unpulsed APCs (APC-0). For the proliferation assay, 50,000 autologous

monocytes were pulsed with peptide, irradiated and co-cultivated with T cells (or T cell clones) at a stimulator : responder ratio of 2:1. After 48 hours, tritiated thymidine was added to cultures at 1 microcurie/well. Cells were harvested after another 18 hours and thymidine incorporation was assayed on a Topcount scintillation counter. The thymidine incorporation in cpm when stimulated with peptide-pulsed APC was 66,000 compared with a count of 1400, with null APC , giving a stimulation index of 66,000/1400 = 47. All results are in triplicate. Clone was tested at a number of stages during isolation and expansion. These results represent the final stage testing before infusion and are representative of other results.



Quality control testing included testing for bacterial, fungal, mycoplasma infection and endotoxin immediately prior to infusion. The clone used for infusion (clone#56) passed all QC testing as specified by the FDA-approved IND for this trial.